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Expression of Mitotic Spindle Checkpoint Protein hsMAD1 Correlates with Cellular Proliferation and Is Activated by a Gain-of-Function p53 Mutant

Yoichi Iwanaga and Kuan-Teh Jeang[1]

Molecular Virology Section, Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Maryland 20892-0460

ABSTRACT

Human mitotic arrest deficiency protein 1, hsMAD1, is a component of the mitotic spindle assembly checkpoint (MSC) that monitors fidelity of chromosomal segregation and guards against emergence of cellular aneuploidy. Because aneuploidy is a pervasive characteristic of human cancers, understanding how MSC genes are regulated is important. Here, we have analyzed human genomic sequences upstream of the 5′ most hsMAD1 coding exon and have identified a 1.5-kb fragment with promoter activity. The *hsMad1* promoter, consistent with characteristics of housekeeping genes, is highly GC rich and is devoid of a TATA-box. Mutational analyses revealed a core region spanning −73 to −31 as being essential for *hsMad1* transcription. Surprisingly, although MSC function, prototypically induced by microtubule inhibitors, is active selectively during mitosis, we found the *hsMad1* promoter to be expressed predominantly in G_1 and to respond not to microtubule inhibitor but to mitogenic stimulus. In primary, as well as transformed cells, intracellular levels of hsMAD1 correlated with the proliferative status of cells. The *hsMad1* promoter was also activated preferentially by a gain-of-function p53 mutant. Taken together, our results suggest that hsMAD1 might link p53 function to the generation of cellular aneuuploidy and that heightened activation of *hsMad1* by gain-of-function p53 mutants could contribute to the worse prognosis of certain cancers.

INTRODUCTION

A portion of all human cancers (70–80%) are aneuploid (1). Indeed, it has been proposed that aneuploidy represents an inciting event for cancer development (2, 3). In eukaryotic cells, a MSC[2] monitors the fidelity of chromosomal segregation during mitosis (4, 5). Disruption of MSC function in mammalian cells has been correlated with aneugenic genomes (6–8). Currently, seven protein components of the MSC have been identified in yeast (MAD1–3, BUB1–3, and Mps1; Refs. 4, 5, and 9), and mammalian cells and have been shown to play roles in MSC (10–15). As yet, no promoter for a mammalian MSC gene has been characterized.

Studies over the last 15 years have revealed the p53 protein to be a critical "guardian of the human genome" (16). Findings of rapid and robust p53 transcription and synthesis after exposure of mammalian cells to genotoxic agents (17–19) have contributed important insights to p53 function. On the basis of this type of evidence, one can infer a feedback linkage between DNA damage and induction of p53, which serves to enforce the repair of genetic lesions (20, 21). Analogously, one could similarly reason that a definition of the signals that provoke expression of MSC proteins would aid in elucidating their functions.

We identified previously and cloned the cDNA for the 718 amino acid human MAD1 protein and showed hsMAD1 to be a binding partner for the 205 amino acid MAD2 protein (10). Although MAD2 has been studied extensively and found to function as a negative regulator of the anaphase promoting complex (22–24), MAD1 has been investigated poorly, and its activities are largely unclear. Toward clarifying its role in cell growth and division, we sought to understand the regulated expression of MAD1 in human cells. Here we describe the cloning and the characterization of the *hsMad1* promoter. Unexpectedly, expression of *hsMad1* was found to not be M but to be G_1 phase specific. Additionally, the *hsMad1* promoter was not responsive to MTI but was activated strongly by phorbol ester. We also found enhanced activation of hsMAD1 expression by a gain-of-function p53 mutant. On the basis of our data, we propose hsMAD1 as a p53 downstream cellular factor that potentially links p53 to aneuploidy in human cells.

MATERIALS AND METHODS

5′ RACE. SMART RACE cDNA amplification kit (BD Clontech) was used to obtain 5′ noncoding sequence of MAD1 cDNA, according to manufacturer's protocol. Briefly, first-strand cDNA was synthesized from human placenta total RNA by oligodeoxythymidylate primers. Then, 5′ RACE fragments were amplified from the template cDNA using the following MAD1 gene-specific primer; 5′-CTGCATGCTCTGCTGGTACTGCATCTGC-3′. The 5′ RACE fragments were TA cloned into pCR2.1-TOPO vector (Invitrogen), and 12 independent fragments were sequenced.

Reporters and Assays. A fragment containing the 5′ most coding exon of hsMAD1 and 1.5-kb upstream nucleotides were amplified by PCR of human genomic DNA using the following primer pairs: 5′-TGGCGTCTTCCATGGGCCGCTCGCAGCCAGCTTGCCGCCGC-3′ 5′-GGGATTAGATCTGGAACAATTAGGGAAGACGTATGGTC-3′. To construct *hsMad1*-promoter reporters, the amplified PCR product was digested with *Bgl*II and *Nco*I and cloned into pGL3-Basic vector (Invitrogen). The resulting construction was confirmed by DNA sequencing. Deletions and mutations of the *hsMad1* promoter were introduced by PCR-based mutagenesis and also confirmed by sequencing. For p53 activation of hsMAD1, HeLa, HCT116, and HCT116p53$^{-/-}$ cells were transfected with *hsMad1* reporter plasmids with either WT or mutant p53 expression vectors using Lipofectamine (Life Technologies, Inc.). Expression vectors for mutant p53 281G and 143A were gifts from Dr. Tslty (25). β-galactosidase reporter pCMVβ (Promega) was used to normalize transfection efficiency. Cells were harvested 24 h after transfection, and luciferase or β-galactosidase activities were measured by using the substrates from Promega or Tropix, respectively. For comparisons of mRNA levels, matched tumor/normal expression array (BD Clontech) was examined to compare hsMAD1 mRNA expression between tumor tissue and the corresponding normal tissue from the same individual, according to manufacturer's protocol. Briefly, the array blot was hybridized with hsMAD1 full-length cDNA probe labeled with α-^{32}P and exposed to X-ray film. Human ubiquitin cDNA probe provided by the manufacturer was used to ensure valid comparisons.

Cell Cultures. The human colorectal cancer cell line HCT116 and the human cervix cancer cell line HeLa were obtained from American Type Culture Collection. HCT116 p53$^{-/-}$ cell line was a gift from Dr. Vogelstein (26). HCT116, HCT116 p53$^{-/-}$, and HeLa cells were maintained in DMEM, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Stable transformants of HeLa or HCT116 cells with the MAD1 1.5-kb promoter luciferase reporter plasmids were selected by 200 μg/ml Zeocin (Invitrogen), and Zeocin-resistant colonies were screened by luciferase assays. Several different clones were isolated and examined as a pool of five different clones for further analyses to exclude the possibility of

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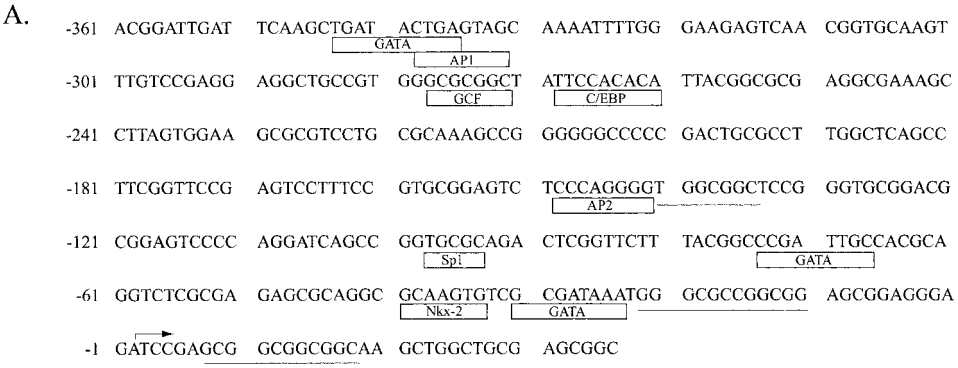
[1] To whom requests for reprints should be addressed, at Building 4, Room 306, 9000 Rockville Pike, Bethesda, MD 20892-0460. Phone: (301) 496-6680; Fax: (301) 480-3686; E-mail: kj7e@nih.gov.

[2] The abbreviations used are: MSC, mitotic spindle assembly checkpoint; MAD, mitotic arrest deficiency; MTI, microtubule inhibitor; RACE, rapid amplification of cDNA end; TNF, tumor necrosis factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; CMV, cytomegalovirus; DAR, daunorubicin; DOR, doxorubicin; NOC, nocodazole; PBMC, peripheral blood mononuclear cell; IL, interleukin; PHA, phytohemagglutinin; WT, wild type; RSC, relaxed spindle checkpoint.

A.



B.

MAD1 promoter	Fold activation+/- SD HeLa	HCT116	HCT116 p53-/-
pGL-3	1	1	1
-1502 to +35	84.86 +/- 10.13	39.10 +/- 5.44	58.91 +/- 5.68
-670 to +35	126.68 +/- 11.88	53.98 +/- 5.14	80.91 +/- 6.93
-487 to +35	169.99 +/- 22.92	46.84 +/- 2.27	80.38 +/- 5.37
-373 to +35	202.49 +/- 29.01	55.58 +/- 2.88	91.63 +/- 13.63
-313 to +35	167.11 +/- 20.23	53.90 +/- 15.35	85.41 +/- 5.17
-193 to +35	153.48 +/- 4.92	41.93 +/- 1.97	66.71 +/- 2.47
-133 to +35	114.01 +/- 12.65	38.36 +/- 3.72	51.56 +/- 9.70
-73 to +35	45.89 +/- 4.98	11.68 +/- 1.02	30.16 +/- 7.55
-31 to +35	0.62 +/- 0.04	0.30 +/- 0.04	0.64 +/- 0.08
+35 to -1502	2.29 +/- 0.30	1.24 +/- 0.11	0.54 +/- 0.10

C.

MAD1 promoter	Fold activation +/- SD
-133 to +35	1
-73 to +35	0.38 +/- 0.11
-31 to +35	0.01 +/- 0.004
mSp1 (-100/-95)	0.58 +/- 0.09
mGATA (-71/-66)	1.07 +/- 0.09
mNkx-2 (-41/-36)	0.42 +/- 0.07
mGATA (-32/-24)	1.09 +/- 0.13
mGC (-23/-12)	0.91 +/- 0.06
mGC (+7/+17)	1.05 +/- 0.10

Fig. 1. Nucleotide sequence of human genomic DNA upstream of the 5′ most human *Mad1* coding exon. In *A*, based on BLAST search of computer sequence database, a 1.5-kb sequence was identified. This sequence was amplified by PCR from human genomic DNA. Within this 1.5-kb fragment, the sequence of the most immediate 360 nucleotides upstream of the major *hsMad1* transcription start site (*arrow*) is shown. The major transcription start site was determined by 5′RACE using human placental RNA as template. Note the absence of a TATA motif and the abundant presence of GC-rich elements. Some putative transcription factor binding sites are boxed. *B*, promoter activity of the 1.5-kb fragment upstream of *hsMad1* coding exons. pGL3-Basic (*top line*) is a control promoterless luciferase plasmid. Into this construction, we positioned various deleted portions of the 1.5-kb MAD1 promoter as indicated. *Bottom line* (+35 to −1502), another control plasmid that contains the 1.5-kb MAD1 promoter inserted into pGL3-Basic in an inverted orientation. Relative luciferase activities from transiently transfected HeLa, HCT116, and HCT116 p53$^{-/-}$ cells, after normalization to cotransfected CMV-β-galactosidase plasmid, are shown. Fold activation is relative to the promoterless pGL3-Basic vector. Error bars were derived from three or more independent experiments. *C*, linker insertion mutagenesis of transcription factor binding sites in the −133 to +34 region of the *hsMad1* promoter. The top three lines (−133 to +35, −73 to +35, and −31 to +35) represent three deleted versions of the *hsMad1* promoter inserted into pGL3-Basic promoter. The remaining six constructions [mSp1 (−100/−95), mGATA (−71/−66), mNKx-2 (−41/−36), mGATA(−32/−24), mGC(−23/−12), and mGC(+7/+17)] represent mutated promoters in which *Eco*RI (gaattc) linkers were substituted, as indicated, into the −133/+34-pGL3-Basic plasmid in a spacing-conservative manner. Luciferase activities from HeLa cells, normalized internally to cotransfected CMV-β-galactosidase plasmid, are presented relative to the −133/+34-pGL3-Basic vector. Error bars were from three or more independent experiments.

clone-specific activities. Cell pools were treated with 100 ng/ml TPA (Sigma Chemical Co.), 20 ng/ml TNF-α (Life Technologies, Inc.), 0.5 μM DAR (Sigma Chemical Co.), 1.01 μM DOR (Sigma Chemical Co.), 200 ng/ml NOC (Sigma Chemical Co.), 5 nM Actinomycin-D (Sigma Chemical Co.), or UV-irradiated (100 J/m^2), and then they were harvested every 3 h for luciferase activities. Primary PBMCs were from anonymous normal donors from the NIH blood bank. Normal foreskin fibroblasts Hs27 and Hs68 were from American Type Culture Collection.

[^{3}H]Thymidine Incorporation. HeLa MAD1-LUC and HCT116 MAD1-LUC cells were cultured without serum for 48 h. The serum-starved, G_0-synchronized cells were then released with 2% serum. 1 μCi of [^{3}H]Thymidine pulsed for 2 h; the cells were harvested every 3 h to measure incorporated [^{3}H].

Computer Analysis for Human Genomic DNA. *Mad1* genomic sequence upstream of the 5′ most coding exon was homology searched by BLAST. A putative transcription start site was identified within an 1.5-kb fragment of upstream sequence by an algorithm for promoter prediction of eukaryotic sequences.[3] *Cis*-elements were predicted by TFSEARCH.[4]

[3] Internet address: http://www.fruitfly.org/seq_tools/promoter.html.
[4] Internet address: http://www.cbrc.jp/research/db/TFSEARCHJ.html.

Western Blotting. Cell lysates were resolved by electrophoresis in 10% polyacrylamide gels and then transferred to Immobilon-P membranes (Millipore). The blots were incubated with anti-β actin antibody (Sigma Chemical Co.), anti-p53 antibody Pab1801 (Santa Cruz Biotechnology), or anti-MAD1 polyclonal serum raised to amino acids 324–498 (10) and visualized by chemiluminescence (Tropix).

RESULTS

Identification of the hsMad 1 Promoter. To search for the *hsMad1* promoter, we queried genomic sequences upstream of the 5′ most coding exon of the human MAD1 cDNA by BLAST homology search. A PAC clone RP5–826E18 from 7p22-p21 (GenBank accession no. AC005282) was identified that contained 98219 nucleotides and had exact identity with the human *Mad1* 5′-exon at positions 8634–8672. Genome sequences upstream of this identity were computer predicted for promoter probability using the TFSEARCH program. On the basis of computer algorithm, a 1.5-kb region at position 7132–8672 of PAC clone RP5–826E18 was suggested to have a high

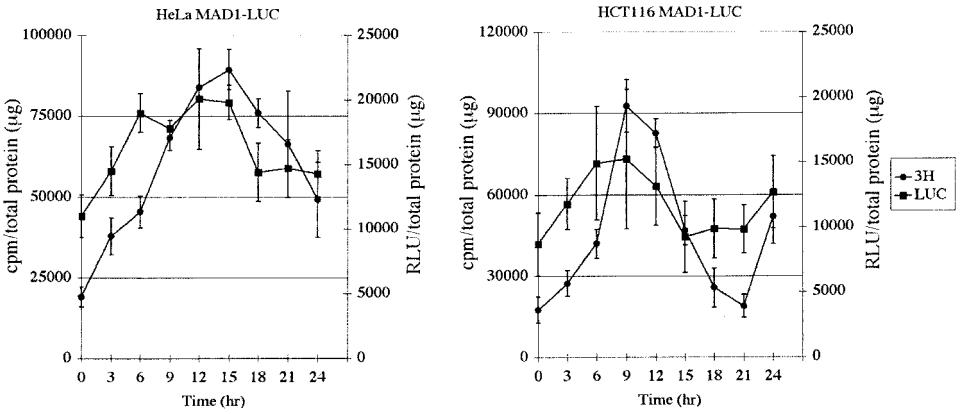


Fig. 2. Cell cycle phase-specific expression of the *hsMad1* promoter. Pools of stable clones of HeLa and HCT116 cells containing the 1.5-kbp *hsMad1* promoter-luciferase reporter (*HeLa M1-LUC* and *HCT116 M1-LUC*) were synchronized by 48 h of serum starvation. Luciferase (*LUC*) activity was determined every 3 h over 24 h after release from serum starvation. Cells were pulse labeled for 2 h at each point with [^{3}H]thymidine ([^{3}H]) to monitor DNA synthesis. Luciferase activities and incorporated [^{3}H]thymidine were normalized to total protein. Data represent average values from three independent experiments. *RLU*, relative luciferase units.

density of transcription factor binding sites consistent with the characteristics of an eukaryotic promoter. Although this stretch of sequence did not contain a prototypic TATA motif, its high GC content (69.8% from −300 to +35) with several GC box motifs (27) is consistent with a promoter for a housekeeping gene (Fig. 1*A*). The computer analysis also predicted a major transcription start site (designated by *arrow*, Fig. 1*A*), which was confirmed experimentally by 5′ RACE analysis of *hsMad1* mRNAs (data not shown).

Definition of Transcriptional Activity from the hsMad1 Promoter. To test whether the 1.5-kb human genomic sequence has promoter activity, we PCR amplified this fragment from HCT116 cellular DNA. Direct sequencing verified that our amplified DNA was identical to the reference human genome sequence. To evaluate transcriptional activity, this 1.5-kb fragment was fused to a firefly luciferase cDNA, and the resulting reporter (−1502 to +35-pGL3 or *hsMad1* promoter; Fig. 1*B*) was tested in transient transfections into three different human cells, HeLa, HCT116, and HCT116 p53$^{-/-}$. Compared with background activity from the promoterless pGL3 luciferase vector, the −1502 to +35-pGL3 plasmid produced 50–100-fold higher luciferase units (Fig. 1*B*). This finding is consistent with the prediction that this fragment contains a housekeeping promoter.

To further define the minimal sequences required for transcription, eight promoter mutations generated by progressively deleting sequences from the 5′ end were constructed. Activities from these *hsMad1* mutant promoters were compared with control pGL3 plasmid, as well as a pGL3 plasmid containing the −1502 to +35 sequence inserted in a reversed orientation (Fig. 1*B*, *top* and *bottom lines*). Luciferase activities were normalized to cotransfected CMV-β-galactosidase reporter. Multiple assays performed separately in HeLa, HCT116, and HCT116 p53$^{-/-}$ cells yielded highly reproducible and consistent values (Fig. 1*B*). Among the deleted promoters, the −670 to +35, the −487 to +35, the −373 to +35, and the −313 to +35 constructions had high luciferase activities. 5′ deletions commencing from either −133 or −73 significantly decreased activity. Further deleting the promoter to a −31 to +35 fragment reduced expression to that indistinguishable from background (Fig. 1*B*). Taken together, the results confirmed a promoter activity for the human 1.5-kb fragment and delineated an essential core promoter that spans positions −73 to +35.

***Cis*-acting Transcriptional Elements in the hsMad1 Promoter.** Next, we systematically queried the relative contribution to activity by various putative transcription factor motifs located between −133 and +35 (Fig. 1*C*). Using *Eco*RI linkers, we individually disrupted by linker substitution the Sp1 (−99/−95), GATA (−75/−68), Nkx-2 (−41/−36), GATA (−32/−24), and GC (−23/−12; +7/+17) elements (Fig. 1*C*). As shown in Fig. 1*C*, disruption of Nkx-2-(GCAAGTG; −41/−36) and the promoter distal Sp1 (TGCGC; −99/−95) motifs reduced basal promoter activity. By contrast, mutation of neither GATA (−32/−24) nor the two transcriptional start site proximal GC boxes (−23/−12; +7/+17) affected promoter function. No individual *Eco*RI linker substitution had the drastic effect on transcription as that produced by truncating the promoter to a −31 to +35 fragment (Fig. 1*C*). These results suggest that multiple factors may interact redundantly within the −133 to +35 region to promote *hsMad1* transcription.

Cell Cycle Phase-specific Expression of hsMAD1. The definition of the *hsMad1* promoter provided an opportunity to investigate its regulated expression during the cell cycle. Because hsMAD1 is an MSC component, *a priori*, one might expect its expression to be coincident with cellular entry into mitosis. To examine this possibility, we derived independent cell clones based on parental HeLa and HCT116 cells (HeLa MAD1-LUC and HCT116 MAD1-LUC), which were transfected stably with the 1.5-kb *hsMad1* promoter luciferase reporter. For cell cycle expression assay, pools of individual HeLa MAD1-LUC or HCT116 MAD1-LUC clones were examined.

Cells were first serum starved for 48 h and then released into complete medium containing 2% fetal bovine serum and sampled every 3 h thereafter for luciferase activity. Duplicate sets of cells were identically processed in parallel except that 1 μCi of [^{3}H]thymidine was added to one set for 2 h before cell sampling. Incorporation of [^{3}H] into acid-insoluble form was monitored for cellular DNA synthesis.

HeLa MAD1-LUC cells, after 48 h of serum starvation, were largely quiescent as reflected by both low luciferase activity and [^{3}H] counts (Fig. 2, *left*). When cells were released from this G_0-like state by exposure to fetal bovine serum, luciferase activity and [^{3}H]incorporation rose progressively. In HeLa MAD1-LUC cells, luciferase values plateaued between 6 and 15 h after release, whereas [^{3}H]incorporation reached a maximum at 15 h and dropped precipitously thereafter (Fig. 2, *left*). Similar profiles were observed in HCT116 MAD1-LUC cells (Fig. 2, *right*). In both HeLa MAD1-LUC and HCT116 MAD1-LUC cells, the peak rise in luciferase activity chronologically preceded the corresponding [^{3}H]curves. The simplest interpretation of these profiles is that *hsMad1* expressed preferentially before the S phase of the cell cycle (*i.e.*, in G_1). Thereafter, the concordant cessation of luciferase activity and [^{3}H]incorporation indicated that the *hsMad1* promoter was minimally active in post-S phases (*i.e.*, in G_2 or M). These results address transcription from the hsMad1 promoter. Elsewhere, we have found that synthesis of the hsMAD1 protein is also under translational and post-translational controls (10).

Mitogen, but not MTI, Activates the hsMad1 Promoter. The G_1-preferred expression of hsMAD1 was an unexpected finding for an

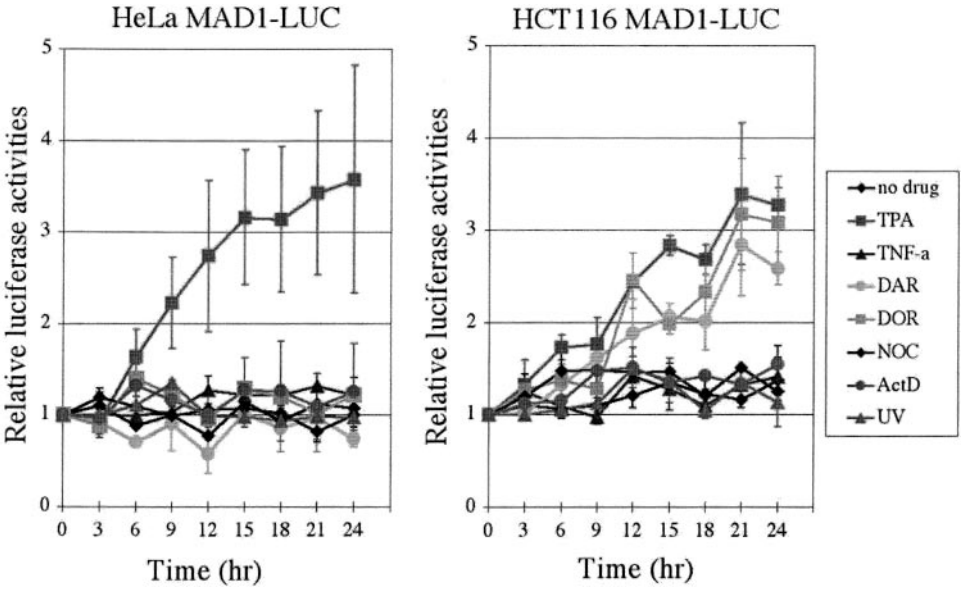


Fig. 3. Responsiveness of the *hsMad1* promoter to various stimuli. HeLa MAD1-LUC and HCT116 MAD1-LUC cells were untreated (*no drug*) or treated with TPA, TNF-α, DOR, DAR, NOC, Actinomycin D, or UV. Cells were harvested every 3 h for luciferase assays. Luciferase activities were normalized to total protein. Data represent average values from three independent experiments.

MSC protein. To further clarify this observation, we sought to understand signals that might activate the *hsMad1* promoter. Therefore, we challenged asynchronously cultured HeLa MAD1-LUC and HCT116 MAD1-LUC cell pools with a variety of stimuli. These included mitogenic stimulator (TPA), stress inducer (TNF-α), DNA-damaging agent (UV light), RNA-synthesis inhibitor (actinomycin D), topoisomerase inhibitors (DAR and DOR), and microtubule inhibitory agent (NOC).

MTIs damage the mitotic spindle and activate MSC function (4, 5). Ordinarily, one might expect NOC treatment to induce *hsMad1* luciferase activity. Surprisingly, neither HeLa MAD1-LUC nor HCT116 MAD1-LUC cells changed in baseline luciferase activity when exposed to NOC (Fig. 3). Indeed, in HeLa MAD1-LUC cells, to the exclusion of all other agents, TPA was the sole treatment that robustly activated the *hsMad1* promoter (Fig. 3, *left*). A similar induction by TPA was also verified in HCT116 MAD1-LUC cells (Fig. 3, *right*). However, here, we found that topoisomerase inhibitors, DAR and DOR, also enhanced *hsMad1* luciferase expression. We do not understand fully what cell-specific differences between HCT116 and HeLa might explain the differential DAR/DOR response; however, independent of this result, both cells demonstrated that the *hsMad1* promoter is strongly stimulated by mitogenic agent, TPA, but not by microtubule disrupting agent, NOC.

Primary Cells Show Reduced Expression of hsMAD1. The above findings prompted us to consider whether MAD1 expression might correlate with the promotion of, rather than the check against, cellular proliferation. To evaluate this hypothesis, we checked the amount of cell endogenous hsMAD1 protein in cells with differing proliferative status. Accordingly, we compared hsMAD1 expression in primary human cells (PBMC, Hs27, and Hs68) and transformed primate cell lines (Jurkat, K562, Cos, CV-1, SW480, and HeLa; Fig. 4).

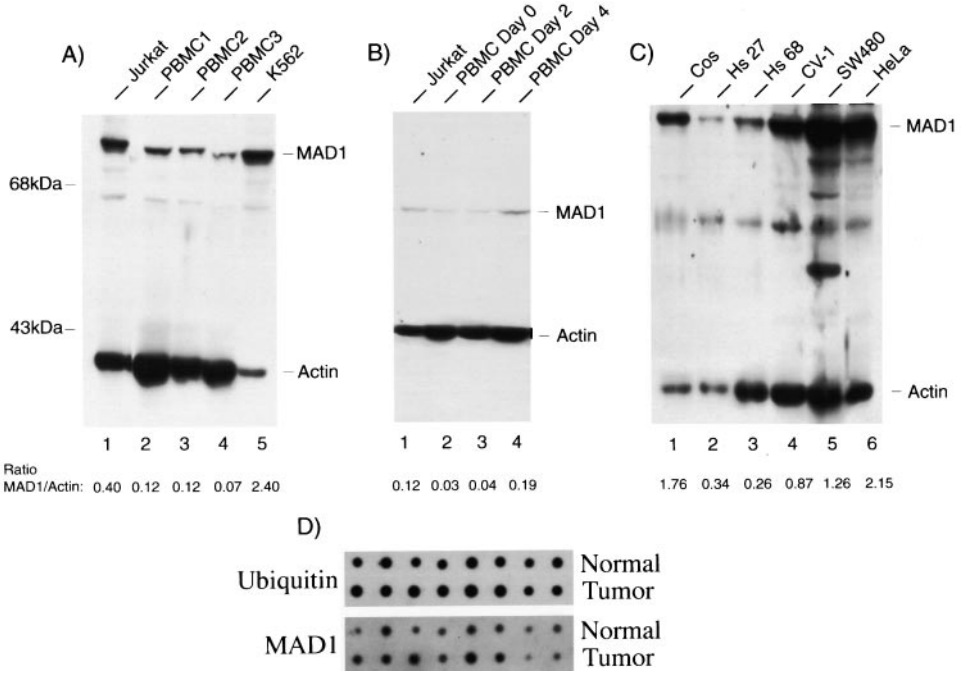


Fig. 4. Levels of hsMAD1 correlated with the proliferation state of the cell. Ambient amounts of hsMAD1 were surveyed in the indicated cells by Western blotting using mono-specific anti-hsMAD1 serum. The relative values of hsMAD1 were normalized internally to β-actin signals (*numbers* at *bottom* of *panels*). *A*, a comparison between three primary PBMCs from normal donors and Jurkat and K562 cells. *B*, a comparison between PBMCs stimulated with IL-2 + PHA and Jurkat cells. *C*, a comparison between two primary foreskin fibroblasts (*Hs27* and *Hs68*) and Cos, CV-1, SW480, and HeLa cells. *D*, a comparison using arrays of normal tumor-matched cDNAs from stomach tissue samples (BD Clontech). The filter array shown at the *bottom* was first hybridized with hsMAD1 cDNA probe and exposed to X-ray film. The same array was then stripped and rehybridized with ubiquitin cDNA probe (*top panel*). Four stomach cancers expressed significantly more *hsMad1* transcripts than their corresponding normal counterparts.

Expression of hsMAD1 in primary PBMCs from three independent normal donors (PBMCs 1, 2, and 3; Fig. 4*A*) was assessed by Western blotting. We internally normalized hsMAD1 from PBMCs to cellular β-actin protein and then compared these values to corresponding values from transformed Jurkat (a human lymphoblastic leukemia cell line) and K562 (a human chronic myelogenous leukemic cell line) cells. In side-by-side comparisons, all three PBMCs conveyed similarly low values for hsMAD1. By contrast, Jurkat and K562 cells had normalized hsMAD1 values that exceeded their untransformed counterparts by 5–20-fold.

Many reasons could explain differences between cell lines and primary cells. To more directly correlate amounts of hsMAD1 with cellular proliferation, we induced PBMCs to proliferate by treatment with IL-2 and PHA. Exposure to IL-2 + PHA activates otherwise quiescent mononuclear cells to divide. When compared with unstimulated PBMCs (Fig. 4*B, Lane 2*), proliferating PBMCs treated with IL-2 + PHA for 4 days (Fig. 4*B, Lane 4*) increased hsMAD1 expression by >6-fold and showed a normalized value that actually exceeded that found for Jurkat cells (Fig. 4*B, Lane 1*). That enhanced hsMAD1 expression correlated with cellular proliferation is fully consistent with above findings (Fig. 3) that the *hsMad1* promoter responds efficiently to mitogenic stimulation.

To extend the generality of the findings beyond PBMCs, we also checked a second type of primary human cells, foreskin fibroblasts. Two independent foreskin fibroblast cultures (Hs27 and Hs68; Fig. 4*C*) were examined. They (Fig. 4*C, Lanes 2* and *3*) were compared with immortalized African green monkey kidney cells (CV-1; Fig. 4*C, Lane 4*), SV40-transformed CV-1 cells (Cos; Fig. 4*C, Lane 1*), and human colon (SW480; Fig. 4*C, Lane 5*) and cervical (HeLa; Fig. 4*C, Lane 6*) cancer cells. In agreement with PBMC findings, both foreskin fibroblasts had much reduced levels of hsMAD1 when compared with the adherent immortalized/transformed cells. Separately, we confirmed that Jurkat and K562 do propagate more rapidly than unstimulated PBMCs and that Cos, CV-1, SW480, and HeLa cells divide faster in culture than either Hs27 or Hs68 (data not shown).

To further evaluate hsMAD1 expression and cellular proliferation, we next examined eight pairs of normal tumor stomach tissue cDNA libraries. The normal tumor cDNA pairs were assessed for MAD1 mRNA levels and then compared after normalizing for control "ubiquitin" mRNA. In four of eight pairs, normalized MAD1 expression was clearly higher in the tumor tissue (Fig. 4*D*), whereas no normal sample had obviously higher MAD1 levels than its tumor counterpart. Taken together with the results in Fig. 4, *A–C*, the data provide a consistent correlation between heightened hsMAD1 expression and increased cellular proliferation.

A Gain-of-Function p53 Mutant Up-Regulated Expression of hsMad1. In considering the hsMAD1 expression profile in cells, we recalled previously that *hsMad1* transcript was 1 of only 14 cellular mRNAs that were induced robustly by a p53-expressing adenovirus vector in colorectal cancer line DLD-1 (28). Although not recognized at the time of publication, the p53-induced gene 9 (*PIG9*) in Polyak *et al.* (28) was, in fact, *hsMad1*. Because other studies have linked aberrant cellular ploidy with p53 mutations (25, 29) and because we observed heightened hsMAD1 expression in cancer cells (Fig. 4), we sought to understand better the possibility that p53 might be an upstream regulator of *hsMad1*. Accordingly, we compared WT p53 and two p53 mutants (143A and 281G; Ref. 25) for respective abilities to activate *hsMad1*. When p53WT, p53 143A, and p53 281G were introduced separately into HeLa cells, ambient expression of hsMAD1 was perturbed minimally by either p53 WT or p53 143A. By contrast, hsMAD1 was increased significantly (≤9.5-fold) by p53 281G in a dose-dependent manner (Fig. 5*A*).

To confirm the ambient expression results, we next checked for effects exerted by the three forms of p53 on the *hsMad1* promoter luciferase reporter. We cotransfected the 1.5-kb *hsMad1* luciferase reporter with p53 WT, p53 143A, or p53 281G, respectively, into HeLa, HCT 116, and HCT 116 $p53^{-/-}$ cells. As shown in Fig. 5*B*, although both p53 WT and p53 143A perturbed expression ≤2-fold, p53 281G induced expression in excess of 6-fold.

To understand how p53 281G activated the *hsMad1* promoter, we separately assayed in HeLa cells the full-length *hsMad1* promoter luciferase plasmid and eight *hsMad1* promoter mutants (Fig. 5*C*). Reproducibly, deletion mutants −133 to +35 and −73 to + 35, and linker insertion mutants −100/−95, −71/66, −41/−36, and −30/−25, maintained responsiveness to p53 281G. By contrast, individual disruptions in two GC boxes (−20/−15 and +9/+14) dramatically abolished p53 281G responsiveness. Interestingly, these two transcription site proximal GC boxes bear resemblance to the canonical 5′ XXXC(A,T)(T,A)GYY 3′ (where X = purines and Y = pyrimidines) p53-responsive motif (30), as well to the p53-responsive GC sequence first characterized for the SV40 GGGCGG sequences (18).

DISCUSSION

MSC monitors fidelity of chromosomal segregation during mitosis. HsMAD1 is a human MSC protein. MAD1 was characterized originally as one of six MSC components in budding yeast (4, 5). Four years ago, we identified the human homologue of yeast MAD1 (10), mapped the gene for *hsMad1* to human chromosome 7 (31), and showed that human MAD1 is the cellular binding partner for MAD2 (10). Although MAD2 has been studied extensively and shown definitively to be an inhibitor of the anaphase-promoting complex (anaphase promoting complex; Refs. 22–24), little is understood about the actions of MAD1 other than that it is required to chaperon MAD2 to kinetochores (32).

Here we sought to characterize *cis*- and *trans*- factors that influence the expression of the *hsMad1* promoter in an attempt to better understand how MAD1 functions. We began with the identification of a 1.5-kb human genomic fragment as the provisional *hsMad1* promoter. Analysis of this sequence revealed it to be TATA-less and highly GC rich with significant promoter activity (Fig. 1). Within this promoter, one can visualize many degenerative forms of the canonical Sp1-GGGCGG-motif or its closely related hexanucleotide GC box sequences (Refs. 27 and 33; Fig. 1). On the basis of the absence of a TATA element and the presence of multiple GC motifs, we interpret the hsMad1 promoter to be characteristic of promoters for housekeeping genes.

In studying the regulated expression of *hsMad1*, we came across several unexpected findings. Contrary to our *a priori* assumption that an MSC protein should be expressed chronologically proximal to the onset of mitosis, we found the *hsMad1* promoter to be basally active in G_2 and M but preferentially active in the G_1 phase of the cell cycle (Fig. 2). Additional observations that spindle disrupting MTI, NOC, failed to induce, whereas mitogenic agent, TPA, did robustly induce the *hsMad1* promoter were also unanticipated (Fig. 3). Add to these results a correlation between levels of hsMAD1 in cells and the proliferation state of the cells (Fig. 4), one is then struck by the possibility that hsMAD1 likely has cellular functions in addition to its expected role in the MSC. Indeed, we do not currently understand the gamut of functions described by MSC proteins. In this vein, it is instructive to note that mice lost for MSC function through "knockout" of the murine *Mad2* gene are embryonically lethal (6). The embryonically lethal phenotype is formally inconsistent with proteins that serve purely an inducible checkpoint function, as exemplified by the normal development to maturity of p53 knockout mice (34).

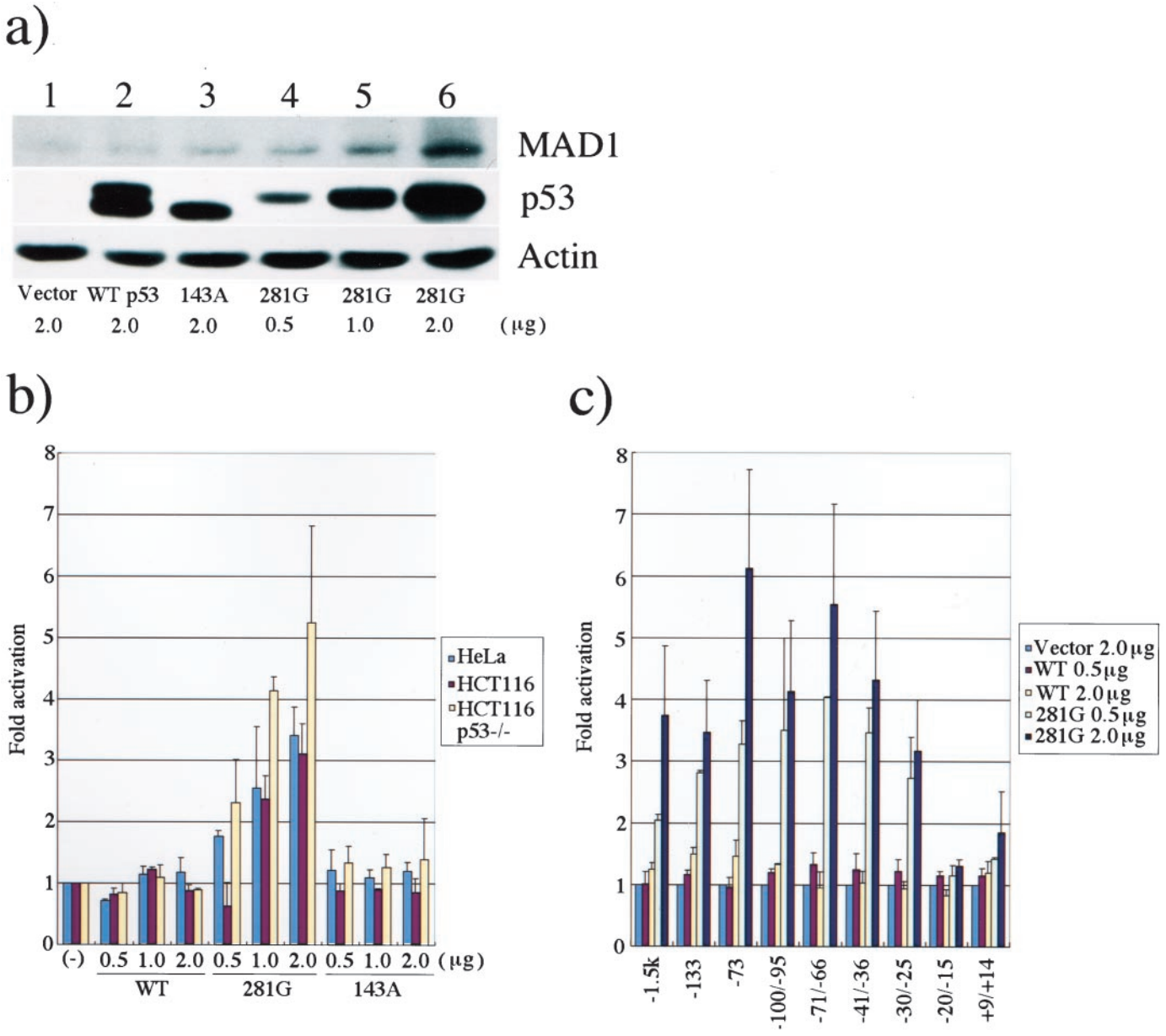


Fig. 5. Preferential activation of *hsMad1* promoter by mutant p53 281G. *a*, HeLa cells were transfected with the indicated p53 expression vector, WT, 143A, or 281G. p53 281G was found to induce MAD1 synthesis in a dose-dependent manner. Amounts of transfected DNA (μg) are indicated at *bottom*. *b*, luciferase assays demonstrating the activation of the 1.5-kb *hsMad1* promoter in HeLa, HCT116, and HCT116 p53$^{-/-}$ cells. *Y* axis shows relative fold activation; *X* axis indicates the various p53 plasmids, as well as the amounts of transfected DNA (μg). In *c*, the p53 281G responsive elements in HeLa cells map to two proximal GC boxes in the *hsMad1* promoter. *Y* axis shows relative fold activation; *X* axis indicates the various *hsMad1* promoter luciferase plasmids. −1.5K (−1502 to +35), −133 (−133 to +35), and −73 (−73 to +35) are promoter-deletion plasmids. −100/−95, −71/−66, −41/−36, −30/−25, −20/−15, and +9/+14 are *Eco*RI linker substitution mutants based in the −133 to +35 promoter luciferase plasmid. Legend indicates control vector (pUC19; 2μg) and the transfected amounts (μg) of the various p53 plasmids.

Hence, our current results coupled with emerging observations[5] that overexpressed hsMAD1 promotes cyclin D expression and cell cycle progression in serum-arrested cells suggest that this protein has function(s) essential for cellular proliferation beyond that needed for MSC activity.

It is intriguing that *hsMad1* is activated by WT p53 (28) and activated even better (in our experiments) by a gain-of-function p53 mutant (Fig. 5). Mutation of p53 is the most common genetic alteration found in human cancers (35), and tumors that express gain-of-function p53 mutants have a worse prognosis than p53-null cancers (36). The tumor-promoting activity of mutant p53 emanates in part from its distinct transactivating ability when compared with WT p53. Thus, several cellular proliferation-related genes, such as *c-myc*, epidermal growth factor receptor, proliferating cell nuclear antigen, and multidrug-resistance-1, have been reported to be activated selectively by mutant p53 but not WT p53 (37). Recognizing that activation of genes by p53 is somewhat cell background dependent (38), within the context of the cells used in this study, our findings suggest that *hsMad1* is another growth-related cellular gene activated preferentially by some p53 mutants. The finding that certain stomach cancers have higher levels of *hsMad1* transcripts (Fig. 4*D*) may indicate the presence of such gain-of-function p53 mutations in those tumors.

Mechanisms as to how mutant p53 transactivates cellular genes appear to be variable and not understood completely. The IL-6 pro-

[5] K-T. Jeang, unpublished observations.

moter, *e.g.*, is transactivated by p53 mutant protein through CAAT/enhancer binding proteinβ motifs (39). In the case of the *hsMad1* promoter, we find it to be activated by p53 281G through two start site proximal GC boxes (Fig. 5), which resemble closely the WT p53-responsive GC sequence described for the SV40 promoter (40). Indeed, our results, consistent with the findings of Polyak *et al.* (28) assayed in a different cellular background, indicate that WT p53 can also slightly activate hsMad1, albeit (in our context) to a level of $<$2-fold (Fig. 5).

In considering the biological relevance of activation by p53, we note the reported linkage between cellular aneuploidy and aberrant p53 function (25, 29, 41). Indeed, interestingly, p53 281G falls into a category of gain-of-function p53 mutants that specify for a RSC phenotype (25). Accordingly, one could view hsMAD1 as a p53-downstream MSC factor whose activity is modulated differentially by p53 mutants. The unexpected finding here that hsMAD1, in addition to its MSC function, has a proliferation-associated role raises the possibility that WT-p53 and RSC-p53 activate divergent hsMAD1 properties. Conceivably, mild *hsMad1* induction represents an attempt by WT-p53, in response to chromosomal mis-segregation, to invoke the MSC function of hsMAD1. On the other hand, robust induction of *hsMad1* by RSC-p53 may activate a proliferative hsMAD1 activity that overrides its MSC function. Pending a better understanding of hsMAD1's non-MSC function(s), additional studies are needed to clarify how the interplay between hsMAD1 and WT, or mutant, p53 might either mitigate or enhance the propensity for development of aneuploidy in mammalian cells.

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